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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/00, G01N 27/327, C12Q 1/34, 1/48, 1/26

A1

(11) International Publication Number: WO 99/07877

(43) International Publication Date: 18 February 1999 (18.02.99)

(21) International Application Number: PCT/GB98/02239

(22) International Filing Date: 5 August 1998 (05.08.98)

(30) Priority Data:

9716453.7	5 August 1997 (05.08.97)	GB
9725231.6	29 November 1997 (29.11.97)	GB

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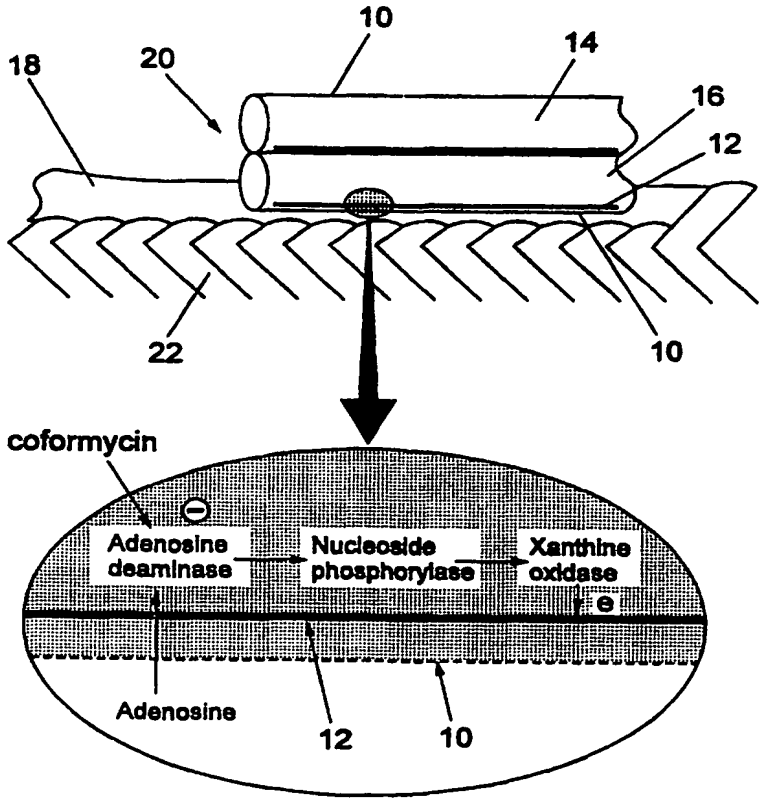
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: BIOSENSOR FOR DETECTING ADENOSINE

(57) Abstract

A biosensor to detect adenosine which comprises the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase, orfunctional equivalents thereof, immobilized on a support and means to detect hydrogen peroxide like an electrolytic cell. Detection of adenosine level can be particularly useful, for example, in the treatment of narcolepsy, heart surgery and to improve the effect of some medications.



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XOCID: <WO___9907877A1_I_>

BIOSENSOR FOR DETECTING ADENSOINE

The present invention relates to a biosensor and assay for detecting adenosine.

Adenosine is an important and near universal neuromodulator in the peripheral and central nervous systems. In the brain adenosine functions to protect cells against ischaemic damage. Additionally, adenosine has been implicated in the regulation of pain pathways, the control of REM sleep, regulation of spinal motor patterns and synaptic plasticity underlying memory. Peripherally adenosine is powerfully regulated in blood plasma and may be involved in regulation of blood pressure and other autonomic functions.

To facilitate study of adenosine various sensitive methods have been developed for measuring adenosine levels. However, such methods ultimately require running a sample through a High Performance Liquid Chromatography (HPLC) machine. Consequently, current methods suffer from the disadvantages due to the absolute requirement for such expensive machinery,

including lack of portability, the necessity of a skilled operator and the time required to perform a measurement. Methodologies reliant upon HPLC techniques also exhibit limited time resolution.

There thus exists a need to develop techniques enabling rapid time resolution of adenosine content in a sample. Desirably, such techniques would involve only portable, inexpensive equipment capable of providing rapid measurements by relatively unskilled operators.

Monitoring of adenosine presence and/or content may be of particular utility in the following situations:

Narcolepsy: is a disorder of REM sleep where the affected individual will experience irresistible sleep attacks of 5 to 30 minutes throughout the day. The incidence of narcolepsy is 0.04 to 0.09% of the population and very often its sufferers go undiagnosed and suffer unwarranted social stigma for apparent laziness. Since adenosine may be involved in turning on REM sleep, it is possible that inadequate regulation of adenosine release could be a contributing factor. This in turn suggests that measurement of adenosine levels in narcoleptics could have diagnostic value.

Effective Medication: many drugs are often only effective if their levels in plasma (and CSF) are kept at therapeutic levels. Elevation of adenosine may be desirable to protect neural damage following stroke, and a suitable measurement method would allow a drug treatment regime to be tailored to achieve the correct levels of adenosine.

Heart Surgery: Adenosine protects the heart during transient oxygen deprivation, by increasing the supply of blood to the heart, and reducing the work performed by the heart. Clinically, adenosine and drugs which target either adenosine degradation or reuptake are used to treat a variety of conditions. Abnormal heart rhythms can be terminated by transient application of adenosine. During heart surgery, the blood supply to the heart muscle is stopped. When the surgery is complete, reperfusion of the heart with blood causes damage to the muscle which can be greatly reduced by treatment with adenosine. However the problem with using adenosine as a treatment is that its actions depend upon the mode and locus of application as well the dose. To compound these problems even further, adenosine has a very short half life in blood (seconds to minutes). Furthermore, if the patients are already on drugs which modify adenosine uptake or degradation there is even further uncertainty over dosage.

The ability to determine adenosine levels in the blood rapidly on-site (e.g. in an operating theatre during surgery or in an Outpatient Department) would remove uncertainty about dosage and would allow optimal treatment with adenosine and thus greatly improve the efficiency of treatment.

EP-A-0184909 to Alberry describes an enzymically based probe which may include the enzyme xanthine oxidase. However, there is no description of a probe capable of monitoring or detecting adenosine.

The present invention provides a bio-sensor comprising

the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase (or functional equivalents thereof) and means to detect hydrogen peroxide. Desirably, the enzymes are in an aqueous environment, for example, are in aqueous solution.

Generally, the enzymes will be entrapped by a suitable means, for example, a semi-porous membrane, although any means which enables the enzymes to interact with substrates in an aqueous phase whilst retaining the enzymes in a particular locality will be suitable. Suitable semi-porous membranes include semi-permeable glass membranes, for example of the type made by Sycopel International. One convenient form of hydrogen peroxide detecting means to be used in the biosensor is an electrolytic cell. It may comprise a single or a dual-barrelled probe each consisting of a 230μm diameter semipermeable cylindrical glass membrane, a working electrode (eg. Pt electrode), a counter electrode (eg. Ag electrode) and a reference electrode (eg. Ag-Agcl electrode). The dual-barrelled probes could be used as a quasi-differential device, in that enzymes can be loaded into only one barrel and the difference signal between the two barrels measured.

When placed into a sample containing adenosine the three enzymes will act in series to convert adenosine to uric acid with the evolution of hydrogen peroxide as a by-product. The rate of production of hydrogen peroxide is therefore proportional to the concentration of adenosine. The hydrogen peroxide can then be detected, for example, by using a platinum electrode. Our experiments have shown that adenosine concentrations as low as 10nM can be detected in this way. One advantage of the bio-sensor of the present invention is that it enables adenosine concentration to

be monitored in real time.

The sequential action of the enzymes involved in the present invention can be described by the following equations which illustrate the order of action of the enzymes:

$$\text{adenosine} \xrightarrow[\text{adenosine deaminase}]{} \text{inosine} + NH_3\uparrow$$

$$\text{inosine} + Pi \xrightarrow[\text{nucleoside phosphorylase}]{} \text{hypoxanthine} + \text{ribose-P}$$

$$\text{hypoxanthine} \xrightarrow[\text{xanthine oxidase}]{} \text{uric acid} + H_2O_2\uparrow$$

The relative concentrations of neighbouring enzymes (ie adenosine deaminase: nucleoside phosphorylase and nucleoside phosphorylase: xanthine oxidase) will affect the efficiency of the bio-sensor since product inhibition may cause a decay in the response observed. Ratios of adenosine deaminase: nucleoside phosphorylase of from 1:100 to 1:1 (especially 1:10 to 1:1) and ratios of nucleoside phosphorylase: xanthine oxidase of from 1:100 to 1:10 (especially 1:50 to 1:10) are satisfactory. In general a relative increase in the concentrations of enzymes used (in the order adenosine deaminase: nucleoside phosphorylase: xanthine oxidase) is required. Examples of suitable such ratios are 1:200:500 which has an efficiency of approximately 50% and 1:2:100 which has an efficiency of approximately 80%. A ratio of adenosine deaminase: nucleoside phosphorylase: xanthine oxidase in the range 1:1:50 to 1:5:200 is preferred and a ratio of approximately 1:2:100 is especially preferred.

In a further aspect, the present invention provides a

method of detecting adenosine in a sample, said method comprising exposing the sample to the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase (or functional equivalents thereof) such that the enzymes can act sequentially on the sample, and measuring the production of hydrogen peroxide. The amount of hydrogen peroxide provided is directly proportional to the amount of adenosine in the sample. If required, the evolution of hydrogen peroxide can be measured over time to enable adenosine content to be monitored, for example, in real time.

In yet a further aspect, the present invention provides a method of diagnosis and treatment of pathological conditions that result from faulty regulation of adenosine, said method comprising detecting the levels of adenosine in a patient in the manner described above. For example the method of the invention can be used in diagnosing sleep disorders (such as narcolepsy).

In still a further aspect, the present invention provides a method of monitoring drug requirements in a patient, wherein said drug affects the *in vivo* levels of free adenosine in a body fluid or an organ of said patient, said method comprising detecting the level of adenosine in said fluid or organ in the manner described above.

In a yet further aspect, the present invention provides a method of monitoring adenosine levels in a patient before, during and/or after surgery, wherein the adenosine levels are detected in the manner described above. A particular example is the monitoring of adenosine levels in the blood supply to the heart at least during part of a cardiac surgical procedure in

order to ensure that, if necessary, adenosine levels are boosted to the levels required to combat damage to the cardiac muscle following reperfusion of the heart. Conveniently the adenosine levels are monitored continuously or intermittently at appropriate time intervals by use of the bio-sensor of the present invention.

In certain samples there may be electro-active species which are also present. These electro-active species could interact non-specifically with the platinum electrode of the bio-sensor and influence the accuracy of the result obtained. Such non-specific interactions should desirably be filtered out from the final reading in order to obtain accurate correlation of hydrogen peroxide production with adenosine content.

In a modification of the method described above, it is envisaged that the biosensor is placed into the sample of interest and a stable reading obtained, this reading being the sum of the interaction at the electrode due to evolution of hydrogen peroxide and also the activity arising from any non-specific electro-active species present. In the modification a specific inhibitor to adenosine deaminase is then introduced. The inhibitor would block the first reaction of the series, preventing hydrogen peroxide production. Consequently, the portion of the final signal due to adenosine presence obtained after inhibitor introduction would cease. In other words, the reduced signal will be due solely to the presence of electro-active species interacting non-specifically with the platinum electrode. This reduced reading would then be subtracted from the initial reading to produce the signal due only to adenosine presence. Suitable inhibitors for adenosine deaminase include EHNA

(erythro-9-(2-hydroxy-3-nonyl)adenine) and coformycin. Further information regarding adenosine uptake systems may be obtained by using a blocker of adenosine uptake, for example NBTG (S-(4-nitrobenzyl)-6-thioguanosine).

The platinum electrode used for hydrogen peroxide detection in the present invention may be connected to a potentiostat which holds the voltage of the electrode constant at +650mV. Suitable equipment is manufactured by Sycopel. It is possible for a reference electrode to be included in the bio-sensor, although this is not essential. A suitable reference electrode could consist of the last two enzymes placed into a buffer solution.

In a further aspect, the present invention provides the sequential use of the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase in a bio-sensor. Generally, the bio-sensor will be adapted to monitor adenosine and will be used in conjunction with a means for detecting hydrogen peroxide.

The technique described above has been used to measure the release of adenosine from *Xenopus* embryo spinal cord during swimming. Adenosine is produced from the ventral part of the spinal cord and builds up slowly during swimming episodes before decaying back to baseline levels once the activity has finished. Our experiments provide the first demonstration that adenosine is released by the spinal cord during motor activity. This is also the first time that adenosine production has been monitored in real time during neural activity.

The invention is further illustrated by the following, non-limiting, examples and drawings:

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. shows the detection of adenosine concentration *in vitro* by enzyme microprobe obtained with a biosensor of the inevntion.

Figure 2. shows the detection of adenosine-release during swimming activity in a *Xenopus* embryo.

Figure 3. shows a schematic representation of the way a biosensor of the invention is working and the biochemical principle behind enzymatic-electrochemical detection of adenosine.

Figure 4. shows *an in vitro* calibration and characterization of an adenosine biosensor of the invention.

Figure 5. shows how a biosensor probe of the invention can detect adenosine released from the spinal cord during fictive locomotion.

Figure 6. shows how blockers of adenosine uptake greatly enhanced the release of adenosine from the spinal cord.

Example 1

Loading biosensor probes with enzyme solutions

0.001U of adenosine deaminase (type VII, SIGMA), 0.2U of nucleoside phosphorylase (from calf spleen, SIGMA), and 5U of xanthine oxidase (from micro-organism, SIGMA) were dissolved in 40μl of a saline consisting of 115mM NaCl, 1mM NaP_i, 10mM HEPES, pH 7.4. 10μl of this solution was then introduced into a biosensor probe (SYCOPEL) at a flow rate of 60μl/hour.

In vitro calibration

A Biosensor Driver (SYCOPEL) was used to hold the probe at +650mV and recorded any current signals generated.

When dual probes were used in a differential mode (with enzymes being present only in one barrel) two Biosensor Drivers were used (one for each probe) and the difference signal between the two was obtained by a differential amplifier. Probes were calibrated by placing them in a continuously stirred bath with a volume of 7ml. Concentrated aliquots of adenosine were successively added to give the desired bath concentration of adenosine. Greatest stability was achieved when the probe and bath were shielded from all air currents. The results are given in Figure 1.

Example 2

Recording adenosine release during swimming in *Xenopus* embryos

Stage 37/38 *Xenopus* embryos were paralysed with α-bungarotoxin and prepared for physiological recordings using well established techniques (eg Dale, N. 1995 "Experimentally derived model for the locomotor pattern generator in the Xenopus embryo" J. Physiol. (Lond.) 489: 489-510). To increase the stability of the recordings, the head and trunk skin of the embryo (which is ciliated and thus causes strong water currents in the bath) was completely removed. The embryos were bathed in a physiological saline that contained 115mM NaCl, 3mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 1mM NaP_i, 2.4mM $NaHCO_3$, 10mM HEPES, Ph 7.4. The muscles overlying the spinal cord were removed and the animal then immobilized in a small recording chamber (0.5ml volume). Extracellular ventral root recordings were made to allow swimming activity to be monitored. The adenosine-sensing probe was carefully aligned with - and gently pressed onto - the lateral side of the spinal cord. There was no fluid flow within the

recording chamber and the chamber and probe were carefully shielded from external air currents. Once a stable background signal had been obtained from the probe, swimming was evoked by brief (0.5ms) electrical stimuli to the tail skin of the embryo. The results are given in Figure 2.

Example 3

Adenosine biosensor probes

Single and dual-barrelled biosensor probes were obtained from Sycopel International. Each barrel consisted of a 230μm diameter semipermeable glass membrane, a Pt working electrode, an Ag counter electrode and an Ag-AgCl reference electrode. They were also fabricated with a 30° bend that allowed the probe to be placed parallel to the embryo spinal cord (see Example 4 below). The dual-barrelled probes could be used as a quasi-differential device, in that enzymes were loaded into only one barrel and the difference signal between the two barrels was measured. In this case the reference and counter electrodes of one barrel were connected to the equivalent electrodes in the other barrel.

0.05U of adenosine deaminase, 0.1U nucleoside phosphorylase (both from calf spleen, SIGMA) and 5U of xanthine oxidase (bacterial, SIGMA) were dissolved in 40μl of saline (115mM NaCl, 1mM NaP_i, 10mM HEPES, Ph 7.4). 10μl of the enzyme mixture was then loaded, at a rate of 30μl/hour, into the probe (one barrel for the dual probes). The probes were controlled by a potentiostat (Biosensor Driver, Sycopel International; one for each barrel for the dual probes) that held the working electrode at +650mV to detect H_2O_2.

For the dual probes the output of the two controlling biosensors was fed into simple differential amplifier to provide a signal that was proportional to the difference between the two probes.

In vitro measurements

Calibration and testing of the probe took place in a vessel (7ml volume). The probe was immersed in saline that was constantly stirred. To ensure maximum stability of measurement, care was taken to shield the vessel and probe from drafts. The adenosine concentration in the vessel was changed by adding concentrated aliquots to raise the overall concentration to known levels. Successive amounts of adenosine were added to give a calibration curve (Fig. 4). Other agents (eg coformycin and inosine were added in this way too).

When loaded into a semipermeable glass microprobe, the three enzymes completed a biosensor (Fig. 3) that was very sensitive to adenosine and showed linear responses from 10nM upwards (Fig. 4a,c,d). In a volume of only a few hundred µl, this is equivalent to a lower limit of detection for adenosine of a few pmol. With complete efficiency in the enzyme cascade, the response to a given dose of adenosine would be identical to that resulting from the same dose of inosine. It was found, by comparing the responses to adenosine and inosine, that the efficiency was around 80% (Fig. 4b). The initial enzyme, adenosine deaminase, can be specifically blocked by coformycin (see Agarwal et al (1978) Methods in Enzymology 51:502-507). Therefore 50-500nM coformycin was added to the bathing medium. This blocked the response to adenosine but crucially had no effect on the response to inosine (Fig. 4b). Coformycin can therefore be used to block only the

first step of the cascade and demonstrate that any responses rely specifically on the activity of adenosine deaminase.

Example 4

The biosensor was next used to monitor the production of adenosine during locomotor activity in the *Xenopus* embryo spinal cord. ATP and adenosine have important actions function on the spinal circuitry (see Dale et al, (1996) Nature 383:259-263) and the changing balance between these two modulators mediates the run-down and spontaneous termination of locomotor activity (see Dale et al (1996) supra). This proposed control system relies on adenosine being produced with a delay from synaptically released ATP so that its build-up throughout motor activity is slow. However direct evidence for the production of adenosine is lacking; it remains unclear whether it is produced from the extracellular breakdown of ATP or is released synaptically; and no information is available about the time course of its production.

Measurements of adenosine release from embryo spinal cord

Stage 37/38 *Xenopus* embryos were prepared for recording by means of well established techniques (Kahn et al (1982) Journal of Experimental Biology 99:185-196). In brief, in accordance with the UK Animals (Scientific Procedures) Act (1986) embryos were anaesthetized in MS222 and the dorsal fin slit. They were then treated with α-bungarotoxin (0.077mg/ml) until they were immobilised. The trunk skin was then removed and the muscles overlying one side of the spinal cord from the hindbrain to the obex were removed to expose the spinal

cord. The animal was pinned in a small chamber (0.5ml volume) so that the lateral side of the exposed cord was uppermost. Ventral root recordings were made from the intermyotome clefts and the biosensor probe was laid along the length of the exposed cord. For dual probes the barrel with enzymes was in contact with the cord, while the reference barrel was necessarily further away (due to the size of the probe relative t the spinal cord, Fig. 3). Thus the dual probe recordings were not true differential recordings. Nevertheless the difference signal was more stable and less prone to drift and environmental disturbance. The ventral root recording and output from the biosensor drivers was plotted on a thermal array recorder (Graftek). Unlike the *in vitro* measurements, the fluid in the recording chamber was kept stationary except during solution changes. The saline for physiological recordings contained 115mM NaCl, 2.4mM $NaHCO_3$, 3mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 1 or 2mM NaP_i, 10mM HEPES, pH 7.4.

When the probe was aligned with the ventral portion of the spinal cord clear responses occurred during motor activity (Fig. 5). The ventral cord also contains the densest staining for 5'-nucleotidase activity. The probe current slowly rose during swimming, and then after the activity had ceased gradually fell back to baseline over a period of several minutes (Table 1). This current was due to release of adenosine from the spinal cord, since block of adenosine deaminase by 50nM coformycin greatly reduced the signal from the probe (n=6). The signals recorded from the probe were variable depending upon the placement of the probe relative to the spinal cord. They corresponded to increases in adenosine concentration ranging from 10nM to 100nM with a mean change of 58nM (n=13, Fig. 5a, Table 1). In 4 additional experiments the change in

adenosine levels was much larger and ranged from 150 to nearly 650nM (mean change 377nM, Fig. 5b, Table 1). These large signals could also be blocked with coformycin (Fig. 5b) and were presumably recorded because the probe was fortuitously placed very close to the source of adenosine production. In these 4 cases, the levels of adenosine continued to rise for 15-72s beyond the end of the episode before falling back to baseline (Table 1). This behaviour may be expected if adenosine is produced from a pool of AMP that accumulates in the extracellular space and persists after neural activity has finished.

Table 1

Type	Change in [Adenosine] (Nm)	Half decay time (sec)	Delay to peak (sec) (peak-end)	Ratio (peak/end)
Small (n=13)	58±6	90±19.3	6.5±4.8	1.1±0.1
Large (n=4)	377±106	104±16.8	49±12.3	2.0±0.4

Magnitude and time course of adenosine-production during swimming. The data are divided into two groups dependent upon size of adenosine response (see text). The "half decay time" is the time for the adenosine level to fall to half its peak value; the "delay to peak" refers to the delay between the end of a swimming episode and the peak of the adenosine response; and the "ratio" is the peak concentration of adenosine divided by that achieved at the end of the episode of swimming. All values expressed as a mean ± sem. The n numbers refer to the number of preparations.

Example 5

To test whether adenosine uptake systems could play a role in limiting the rise of adenosine during locomotor

activity, the effects of NBTG a blocker of adenosine uptake, were studied. At 1μM, NBTG had two effects (Fig. 6): it greatly enhanced the magnitude (means 60 ± 9 and 175 ± 44 Nm in control and NBTG respectively, n=5) and rate of the rise in adenosine concentration (means 37 ± 7 and 101 ± 34 $Nm.min^{-1}$ in control and NBTG respectively, n=5); and it slowed the recovery after the cessation of motor activity (in 3 of 5 preparations the probe signal did not decay to half peak within 5 minutes). This result suggests that adenosine uptake plays an important role in slowing and limiting the rise in adenosine concentrations during activity.

That levels of adenosine can continue to rise even after locomotor has ceased, effectively rules out the possibility that adenosine is released from neurons as a transmitter. Instead, it strongly suggests that it is produced from the breakdown of synaptically released ATP via an extracelluar intermediate. The possible time course of ATP catabolism was analysed by modifying a model for ectonucleotidase action that was originally proposed for endothelial cells (see Gordon et al. (1986) Journal of Biological Chemistry 261: 15496-15504). This earlier work used Michaelis-Menten kinetics to describe the actions of each enzyme, and incorporated feed-forward inhibition by ADP of the conversion of AMP to adenosine as described below.

Simulation of breakdown of ATP

The methods and equations of Slakey (1986) Journal of Biological Chemistry 261: 15505-15507 were adapted. In brief, the breakdown of ATP was considered as 4 coupled irreversible reactions (through ADP, AMP and finally adenosine). The velocity of each reaction (without feed-forward inhibition) was described by the following equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

The four coupled reactions were:

$$\frac{d[ATP]}{dt} = -v_{ATP} + k_R \quad (2)$$

$$\frac{d[ADP]}{dt} = v_{ATP} - v_{ADP} \quad (3)$$

$$\frac{d[AMP]}{dt} = v_{ADP} - v_{AMP} \quad (4)$$

$$\frac{d[ADO]}{dt} = v_{AMP} - v_U \quad (5)$$

where k_R is the rate of release of ATP (and was set to 3 during swimming and 0 at other times); v_{ATP}, v_{ADP} and v_{AMP} are the velocities of breakdown of ATP, ADP and AMP and v_U is the velocity of adenosine-uptake. The velocities v_{ATP} v_{ADP} and v_U were calculated according to equation (1). However, to incorporate competitive inhibition by ADP of the breakdown of AMP, v_{AMP} was described by the following equation:

$$v_{AMP} = \frac{V_{max}[S]}{K_m\left(1+\frac{[ADP]}{K_i}\right)+[S]} \quad (6)$$

where K_i is the equilibrium constant of inhibition. The parameters used are taken from Slakey et al and are summarized in Table 1. The four differential equations (2-5) were integrated numerically using a Runge-Kutta fourth order algorithm with adaptive step size control (see Press et al (1988) Numerical recipes in C. The art of Scientific computing Cambridge University Press). Simulations were run on a Sun Ultra 170E.

Without feed-forward inhibition of the breakdown of AMP, the peak of adenosine concentration occurred close to the end of the episode of activity (Fig. 6b).

However when feed-forward inhibition was introduced, AMP accumulated during the activity and the build-up of adenosine was slowed and its peak concentration was delayed until well after the end of activity (Fig. 6c, compare to Fig. 4b). These new observations directly demonstrate that adenosine is produced from ATP in the extracellular space and strongly support the existence of feed-forward inhibition to slow the build-up of adenosine. This suggests, in turn, that the run-down of motor activity depends very strongly on the nature of the feed-forward inhibition of the 5'-nucleotidase.

A period of relative refractoriness for motor activity follows swimming episodes in the *Xenopus* embryo. To reliably elicit episodes of consistent length, a gap of at least 3 minutes must elapse between the end of one episode and the onset of the next (see Wall and Dale (1995) Journal of Physiology 487: 557-572). As this period correlates well with the elevated levels of adenosine that follow an episode of swimming, the persistence of adenosine in the extracellular space may contribute to the transient refractoriness of spinal circuits following motor activity.

This new method could be adapted to allow real-time measurement of adenosine production both in brain slices and freely behaving animals. In both cases the ability to perform rapid determination of adenosine levels and specifically relate any changes to neural activity should greatly enhance our understanding of the functional roles of adenosine. This technique could be used in a device capable of the rapid determination of adenosine in human blood and CSF which may be of value in the diagnosis and treatment of disorders of the heart and circulation, asthma and neurological deficits resulting from faulty regulation

of adenosine.

Table 2

	ATP	ADP	AMP	Adenosine uptake
V_{max}	22	3.2	3.0	1
K_m	333	95	9.4	10
K_i	-	3.3	-	-

Kinetic parameters used in model for simulation of breakdown of ATP. Units for V_{max} are arbitrary while those for K_m and K_i are in μM.

FIGURE LEGENDS

Figure 1 - Detection of adenosine *in vitro* by enzyme microprobe

A A dual probe was run in quasi-differential mode with enzymes present in only one barrel. The difference signal between the two probes is plotted against time as successive additions to adenosine raise the bath concentration of adenosine to 10, 20, 40, 80 and 160nM.

B Plot of the peak current response versus concentration of added adenosine. The response is linear and has a slope of 3.6 nM/pA.

Figure 2 - Detection of adenosine-release during swimming activity in a *Xenopus* embryo

A Top trace (probe) is the signal from the dual probe in differential mode. The bottom trace (v.r.) is the ventral root activity recorded from a paralysed embryo. Although the embryo is paralysed it can still produce the appropriate neural commands to control swimming and these are monitored by the ventral root electrode. Swimming

activity was elicited by an electrical stimulus to the skin at *. The episode lasts nearly 3 minutes before spontaneously stopping. During swimming the signal from the adenosine probe gradually rises. Once the episode of swimming finishes, the signal from the probe falls back to baseline.

B The specific signal related to adenosine can be blocked by EHNA, an inhibitor of adenosine deaminase. In the same preparation as (A) EHNA was added to the bath and swimming evoked. Only a much smaller, non-specific signal is seen.

Figure 3. The principle behind the enzymatic-electrochemical detection of adenosine.
Schematic of a dual biosensor probe 20 lying parallel to the spinal cord 18 (drawn roughly to scale, Top). Inside one barrel 16, the three enzymes of the cascade are present. Inside the other barrel 14, no enzymes are present. Adenosine diffuses from myomers 22 through the semipermeable glass membrane 10 and is successively metabolized to uric acid with the liberation of H_2O_2 which then donates electrons to the Pt working electrode 12 at which is applied a voltage of +650mV. The current detected is thus proportional to the amount of adenosine present.

Figure 4. *In vitro* calibration and characterization of the adenosine biosensor.
(a) Successive amounts of adenosine were added to the bath at each arrow to raise the concentration of adenosine in the bath by the amount indicated under each arrow. The change in probe current resulting from each application of adenosine is plotted in (c). This shows that probe responds in a linear fashion.

(b) In the same experiment 80nM inosine was added (immediately after the 80nM adenosine). The response to inosine (substrate for the second enzyme in the cascade) was about 25% bigger than the response to the same amount of adenosine, indicating some loss of efficiency in the probe. 500nM coformycin, a specific blocker of adenosine deaminase was added. This rapidly reduced the probe current (due to the continued presence of adenosine in the bath) and greatly attenuated the response to subsequent addition of adenosine. However the response to inosine was unaffected. Thus coformycin only disables the first part of the cascade but leaves the rest intact making it a good test for the specificity of the device.

(d) After the coformycin had been washed out, the sensitivity of the probe to adenosine recovered (although it was still slightly lower than in c). This calibration shows that the response to adenosine was linear from 10nM to 2μM.

Figure 5. The biosensor probe can detect adenosine released from the spinal cord during fictive locomotion.

(a) Production of adenosine during two consecutive episodes of swimming monitored by a ventral root recording (v.r.). Note the slow rise in the probe current and the slow decay after the end of swimming episode. The increases in probe current are equivalent to a change in adenosine concentration of about 60nM. The record at the right shows that application of 50nM coformycin blocks most of the probe current indicating that the signal is largely due to the release of adenosine.

(b) Example (from another preparation) where favourable

placement of the probe relative to the spinal cord resulted in a massive signal equivalent to a change in adenosine concentration of about 370nM. In this case there is a fast component to the probe current (arrow) seen at the beginning of the swimming activity. Note that the probe signal continues to rise for about 50s after the end of the swimming episode. Application of 50nM coformycin blocked the probe current, but left a small fast component. The large slowly developing component of the probe current was thus specifically due to the release of adenosine.

Figure 6. Blockers of adenosine uptake greatly enhanced the release of adenosine from the spinal cord. (a) In the control (left) the probe current involved both fast (arrow) and slow components, the slow component being equivalent to a rise in adenosine concentration of around 64nM. After application of 1μM NBTG (right) to block adenosine-uptake, the fast component (arrow) was unchanged, but the slow component was greatly increased in amplitude and rate of rise (equivalent to a change of about 150nM).

(b) Simulation of the breakdown of ATP without feed-forward inhibition by ADP. The peak of adenosine concentration is only lightly delayed relative to the end of a swimming episode (shown by bar).

(c) When feed-forward inhibition is incorporated, AMP accumulates and the peak of adenosine concentration occurs well after the cessation of activity. The trace for ATP is unmarked in both panels. Both the concentration and time scales are in arbitrary units.

CLAIMS

1. A biosensor to detect adenosine which comprises the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase, or functional equivalents thereof, immobilized on a support and means to detect hydrogen peroxide.

2. A biosensor as claimed in Claim 1, wherein said detection means comprises an electrolytic probe.

3. A biosensor as claimed in Claim 1 or 2, wherein said enzymes are in an aqueous environment.

4. A biosensor as claimed in any of Claims 1 to 3, wherein said enzymes are immobilised on a semi-porous membrane.

5. A biosensor as claimed in Claim 4, wherein said semi-porous membrane is a semi-permeable glass membrane.

6. A biosensor as claimed in any of Claims 1 to 6, which comprises a single or a dual-barrelled probe, said probe consisting of a glass membrane, an electrolytic cell which comprises a working electrode, a counter electrode and a reference electrode and wherein the said enzymes are immobilized onto said glass membrane.

7. A biosensor as claimed in Claim 6, wherein

said working electrode is platinum, said counter electrode is silver and said reference electrode is a silver/silver chloride type electrode.

8. A biosensor as claimed in either of Claims 6 and 7, wherein said glass membrane is cylindrical in shape and has a diameter ranging from about 200 and 300μm.

9. A biosensor as claimed in any of Claims 6 to 8, wherein said probe is a dual-barrelled probe which is used as a quasi-differential device and wherein said enzymes are immobilized on only one of the barrels and the difference signal between the two barrels measured.

10. A biosensor as claimed in any of Claims 1 to 9, wherein the ratio of adenosine deaminase: nucleoside phosphorylase is in a range from 1:100 to 1:1, especially 1:10 to 1:1, and wherein ratios of nucleoside phosphorylase: xanthine oxidase is in a range from 1:100 to 1:10, especially 1:50 to 1:10.

11. A biosensor as claimed in Claim 10, wherein the ratio of adenosine deaminase:nucleoside phosphorylase:xanthine oxidase is in a range from 1:1:50 to 1:5:200.

12. A biosensor as claimed in Claim 11, wherein the ratio of adenosine deaminase:nucleoside phosphorylase:xanthine oxidase is approximately 1:2:100.

13. A method of detecting adenosine in a sample, said method comprising exposing the sample to the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase, or functional equivalents thereof, such that the enzymes can act sequentially on the sample, and measuring the production of hydrogen peroxide there from.

14. A method of monitoring the amount of adenosine in an patient which comprises repeatedly measuring the amount of hydrogen peroxide in a patient over time according to the method as claimed in Claim 13.

15. A method of diagnosis and treatment of pathological conditions that result from faulty regulation of adenosine, said method comprising detecting the levels of adenosine in a patient by exposing at least one sample of said patient to the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase, or functional equivalents thereof; such that the enzymes can act sequentially on the sample; and measuring the production of hydrogen peroxide there from.

16. A method of monitoring drug requirements in a patient, wherein said drug affects the *in vivo* levels of free adenosine in a body fluid or an organ of said patient, said method comprising detecting the level of adenosine as described in Claim 13.

17. A method of monitoring adenosine levels in a patient before, during and/or after surgery,

wherein the adenosine levels are detected according to the method described in Claim 14.

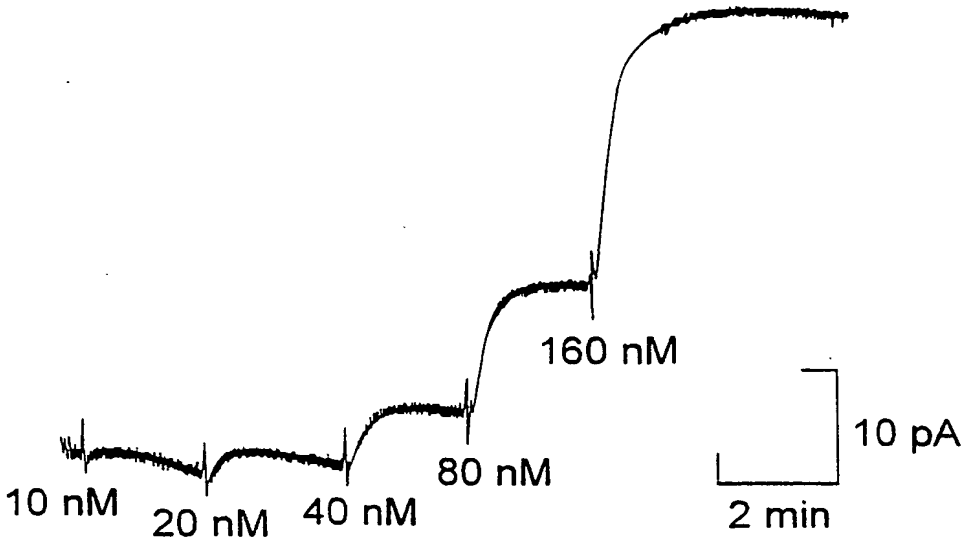
160 nM
10 pA
80 nM
10 nM
20 nM
40 nM
2 min

Fig. 1A

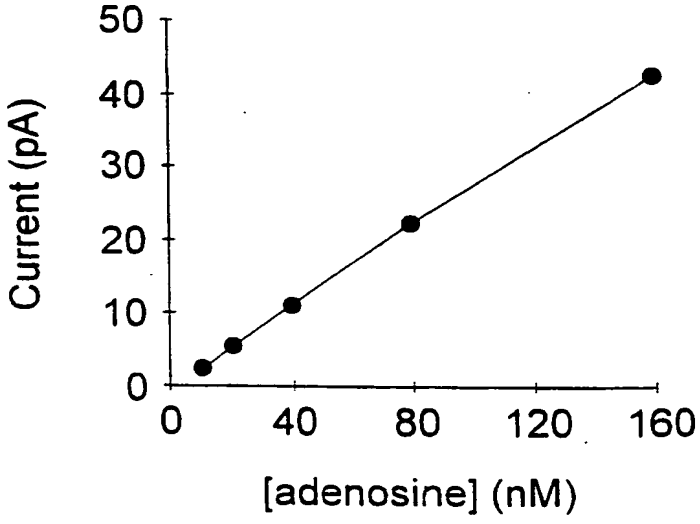
50
40
30
20
10
0
Current (pA)
0
40
80
120
160
[adenosine] (nM)

Fig. 1B

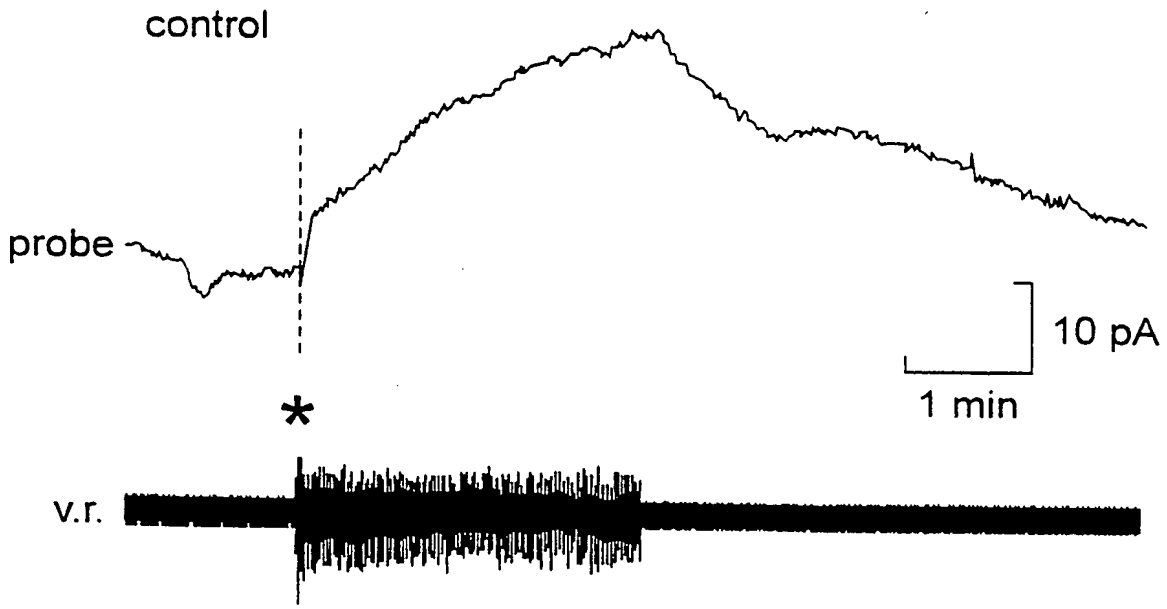

Fig. 2A

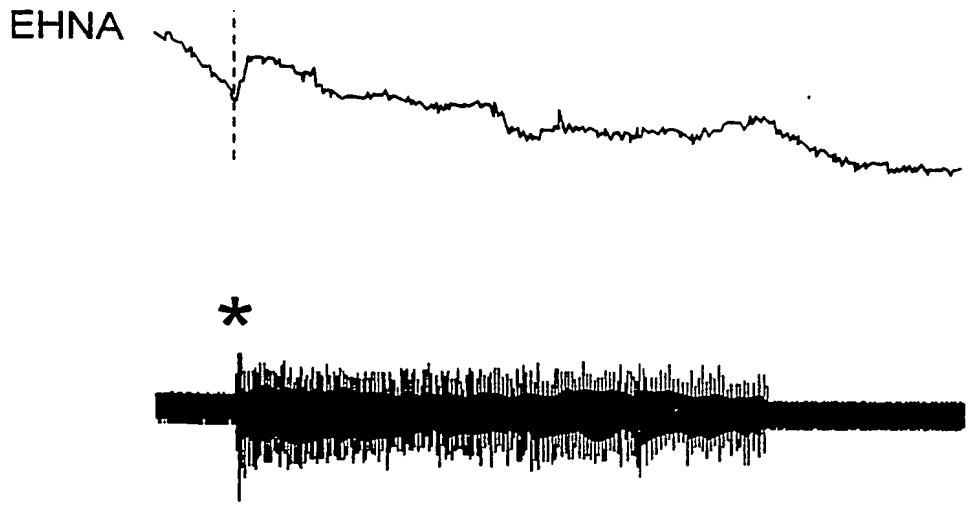

Fig. 2B

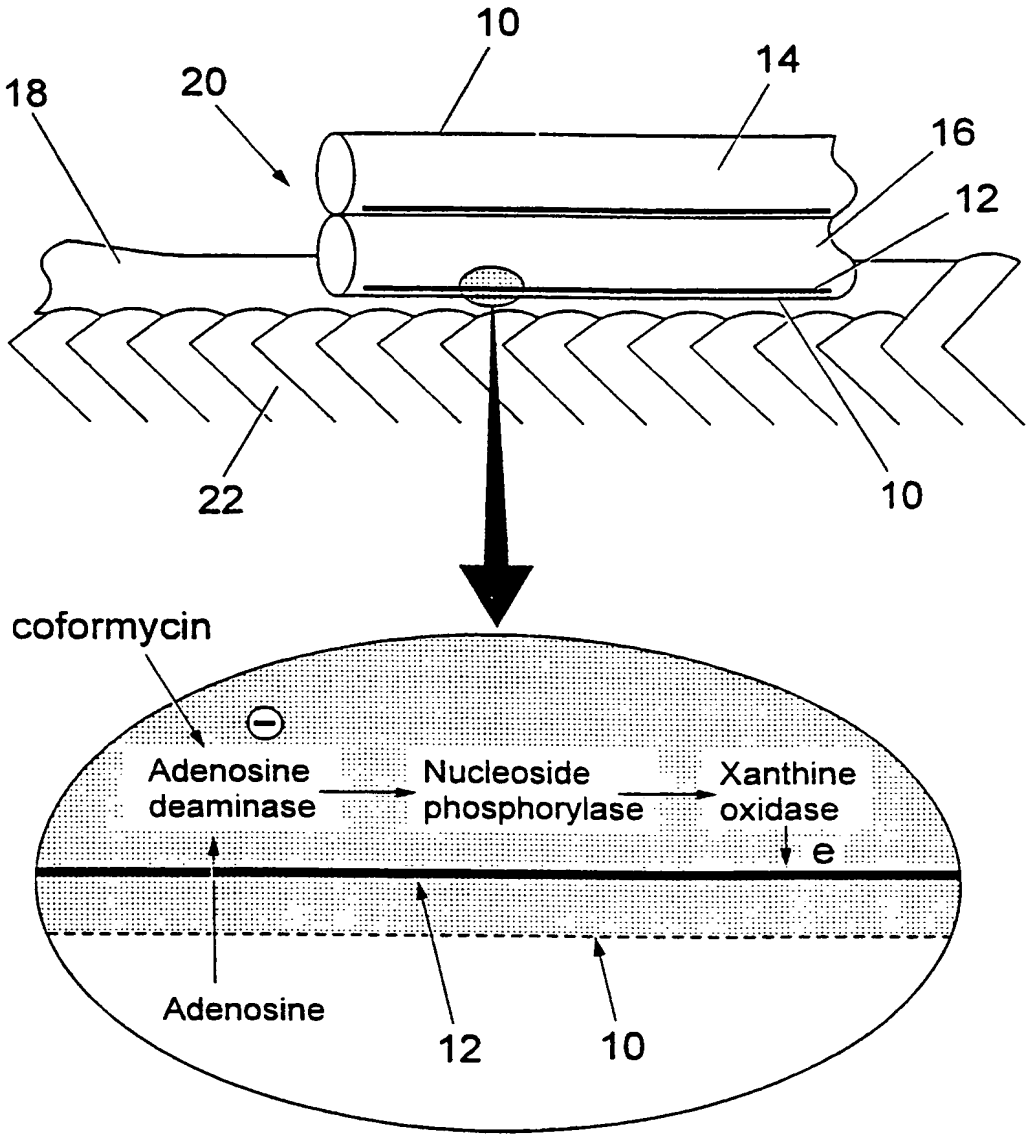
10
14
18
20
16
12
22
10
coformycin
Adenosine deaminase
Nucleoside phosphorylase
Xanthine oxidase
e
Adenosine
12
10

Fig. 3

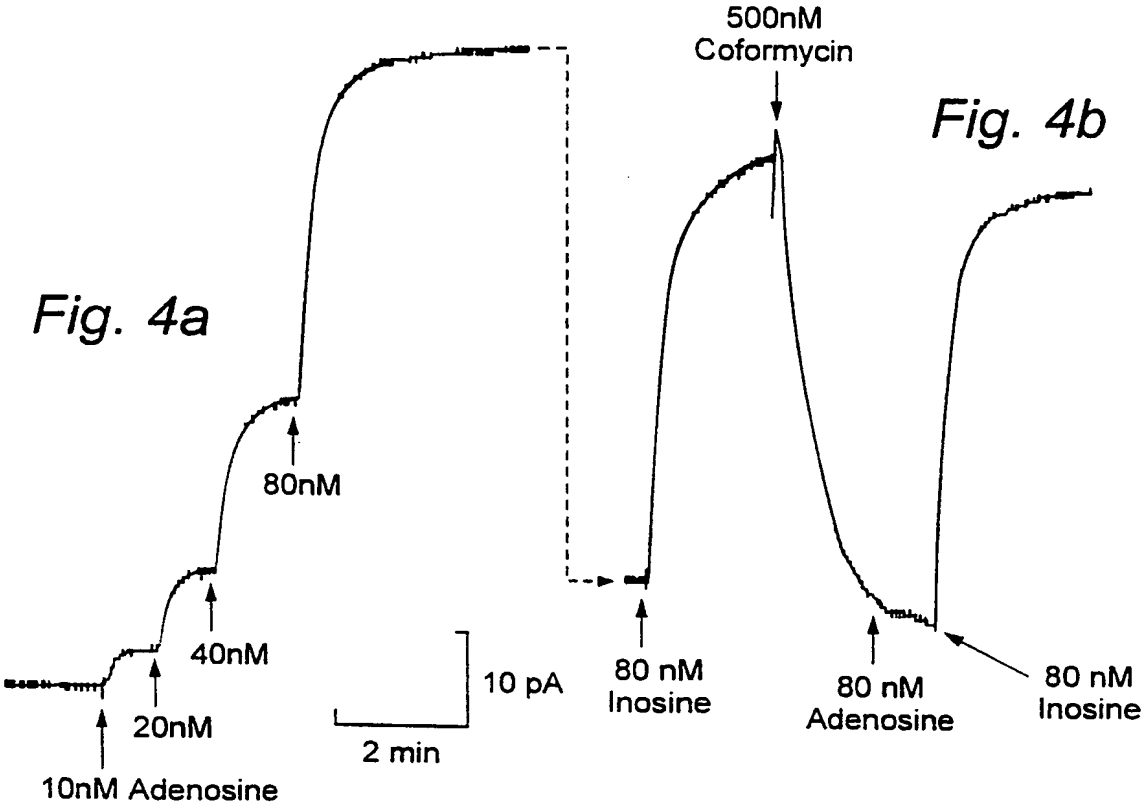

Fig. 4a

Fig. 4b

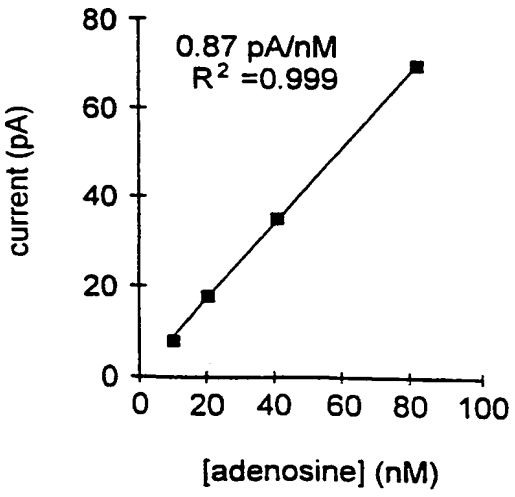

Fig. 4c

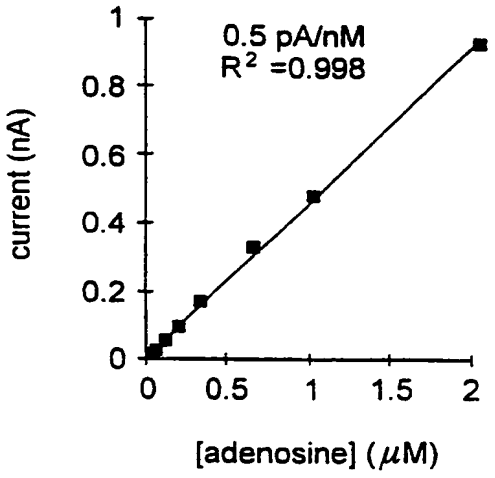

Fig. 4d

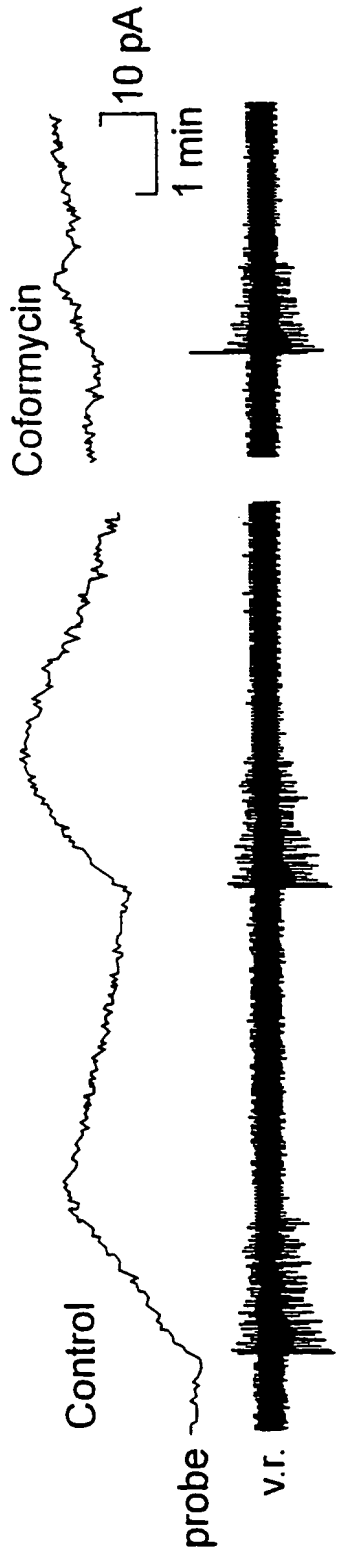
Control
Coformycin
10 pA
1 min
probe
v.r.

Fig. 5a

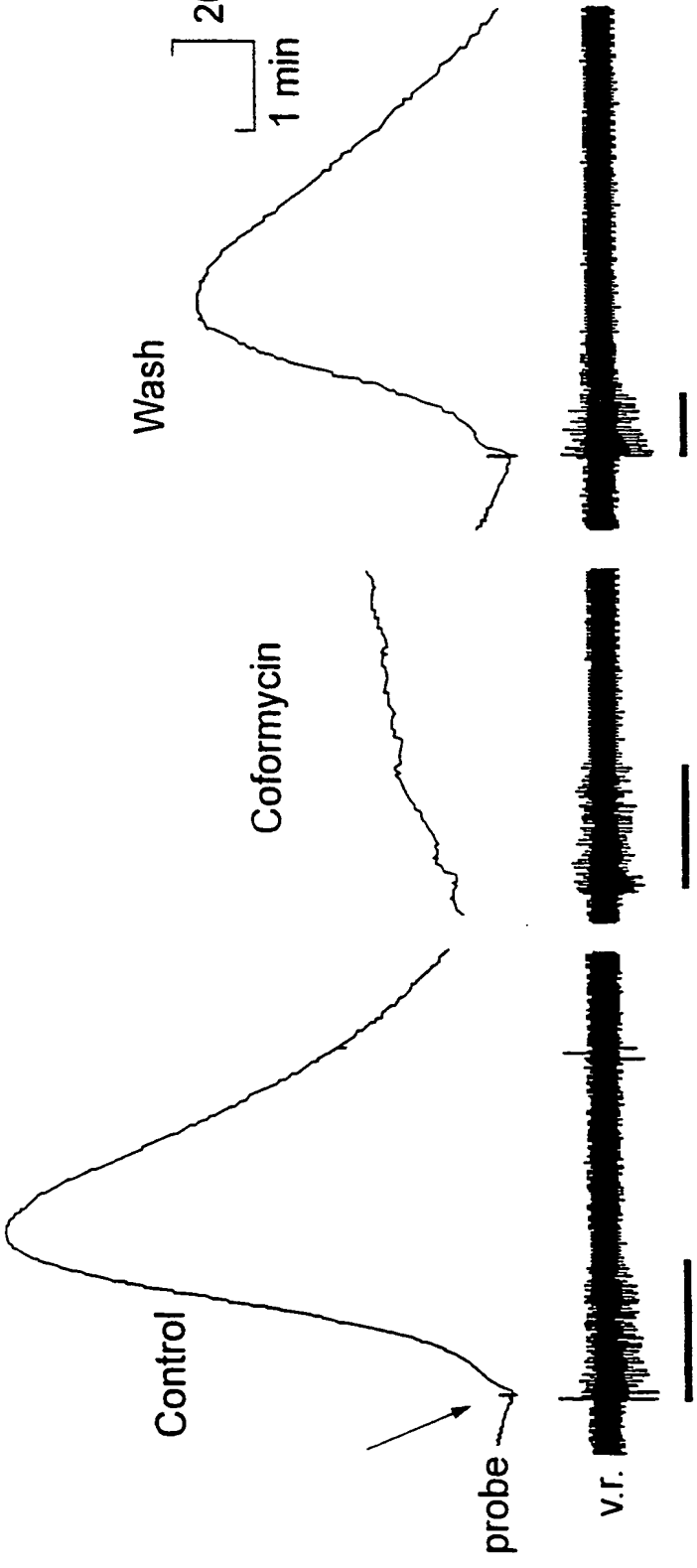
Wash
20 pA
1 min
Coformycin
Control
probe
v.r.

Fig. 5b

Fig. 6a

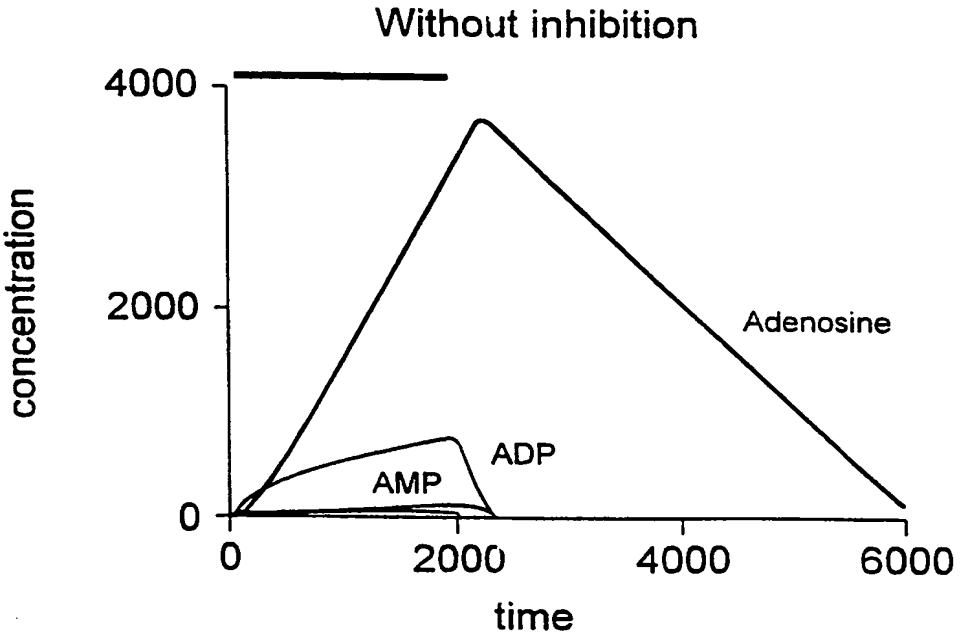
Without inhibition
4000
2000
0
concentration
Adenosine
ADP
AMP
0
2000
4000
6000
time

Fig. 6b

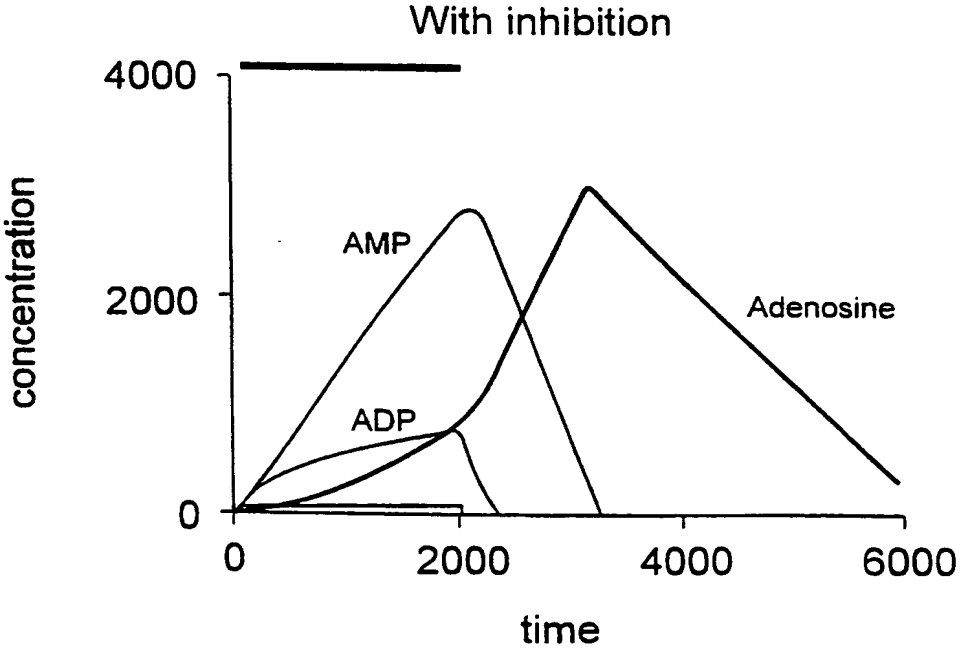
With inhibition
4000
2000
0
concentration
AMP
Adenosine
ADP
0
2000
4000
6000
time

Fig. 6c

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/02239

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/00 G01N27/327 C12Q1/34 C12Q1/48 C12Q1/26

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 288 613 A (LUONG JOHN H T ET AL) 22 February 1994 see column 3, line 56 - column 4, line 60; claim 10 see abstract	1-13
X	PATENT ABSTRACTS OF JAPAN vol. 096, no. 012, 26 December 1996 & JP 08 205891 A (NEW JAPAN RADIO CO LTD), 13 August 1996 see abstract	1-5

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 1998	05/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/02239

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 8808 Derwent Publications Ltd., London, GB; Class B04, AN 88-054472 XP002081753 & JP 63 011848 A (ORIENTAL ELECTRIC CO LTD), 19 January 1988 see abstract	1-5
X	Y. HAYASHI ET AL.: "Flow-injection determination of adenosine and inosine in blood plasma with immobilized enzyme columns connected in series and fluorimetric detection." ANALYTICA CHIMICA ACTA, vol. 186, 1986, pages 131-137, XP002081750 see the whole document	1,13
P,X	C. D. T. BRATTEN ET AL.: "Single-cell measurements of purine release using a micromachined electroanalytical sensor." ANALYTICAL CHEMISTRY, vol. 70, no. 6, 15 March 1998, pages 1164-1170, XP002081751 COLUMBUS US see abstract	1,13
A	US 5 534 504 A (SOLLEVI ALF) 9 July 1996 see the whole document	15
A	H. KATHER ET AL: "Chemiluminescent determination of adenosine, inosine, and hypoxanthine/xanthine." ANALYTICAL BIOCHEMISTRY, vol. 163, no. 1, 1987, pages 45-51, XP002081752 see the whole document	1,13

1

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/GB 98/02239

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14-17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14-17 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/GB 98/02239

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5288613 A	22-02-1994	CA 1312117 A	29-12-1992
		JP 2010155 A	12-01-1990
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		EP 0506205 A	30-09-1992
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		JP 63501497 T	09-06-1988
		WO 8701593 A	26-03-1987